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ADJUVANT ACTION OF LIPOPOLYSACCHARIDES AND DERIVATIVES

Subtitle: Newer Aspects of the Adjuvant Action of Lipid A and Its Analogs

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Airlie Conference Center • Airlie, Virginia • April 6-9, 1991

Sponsored By:

Vaccine Research and Development Branch, Division of AIDS
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Agenda

NEWER ASPECTS OF THE ADJUVANT ACTION OF LIPID A AND ITS ANALOGS

Airlie Conference Center
Airlie, Virginia
April 6-9, 1991

AGENDA

Saturday, April 6

2:00 noon - 6:00 p.m.	Registration	
7:25 p.m. - 7:30 p.m.	**Welcome Notes**	Dr. Arthur G. Johnson
7:30 p.m. - 8:15 p.m.	**Keynote Address** *Current Developments in AIDS Vaccine Adjuvant Research*	Dr. Wayne C. Koff
:30 p.m.	Reception	

Sunday, April 7

8:30 a.m. - 12:10 p.m.	**Session I** **New Advances in the Structural and Functional Relationships of Lipid A and Its Analogs (Part I)**	*Chairperson:* Dr. Ernst Rietschel
8:30 - 8:50	*Overview*	Dr. Ernst Rietschel
8:50 - 9:35	*Lipid A Analogs Aiming at Prevention of the Deleterious Effects of Endotoxin: Structure-Activity Relationships*	Dr. Peter Stuetz
9:35 - 10:20	*Biological Activities of Nonpyrogenic Synthetic Lipid A Analogs with Monosaccharide Backbone*	Dr. Motohiro Matsuura
10:20 a.m. - 10:40 a.m.	Coffee Break	
10:40 - 11:25	*Current Status of the Structure-Function Relationships of Lipid A*	Dr. Kuni Takayama
11:25 - 12:10	*Regulation of Nitric Oxide Synthesis by Lipid A Derivatives*	Dr. Gary L. Gustafson
12:15 p.m. - 1:30 p.m.	Lunch	

Sunday, April 7 (continued)

1:30 p.m. - 4:50 p.m.	**Session II** **New Advances in the Structural and Functional Relationships of Lipid A and Its Analogs (Part II)**	*Chairperson:* Dr. Alois Nowotny
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2:15 - 3:00	*Modulation of Antibody Isotype by LPS and Nonionic Block Copolymers*	Dr. Robert L. Hunter
3:00 p.m. - 3:20 p.m.	Coffee Break	
3:20 - 4:05	*Lipid A-Like Molecules Antagonize the Effects of Endotoxins on Human Monocytes and Neutrophils*	Dr. Douglas Golenbock
4:05 - 4:50	*Host Responses to LPS and Bacteria*	Dr. Christopher Galanos
7:30 p.m.	**Evening Workshop** **Implications and Consequences of Lipid A Administration**	*Chairperson:* Dr. Carl R. Alving

Monday, April 8

8:30 a.m. - 12:10 p.m.	**Session III** **Cellular Mechanisms and Cytokines**	*Chairperson:* Dr. Phillip J. Baker
8:30 - 9:10	*Inactivation of Suppressor T Cell Activity with MPL and Its Analogs*	Dr. Phillip J. Baker
9:10 - 9:50	*Immunoregulation by Lipid A Analogs*	Dr. Arthur G. Johnson
9:50 - 10:30	*Early Endotoxin Tolerance: A Model for Differential Cytokine Induction and Gene Expression by LPS and MPL*	Dr. Stefanie N. Vogel
10:30 a.m. - 10:50 a.m.	Coffee Break	
10:50 - 11:30	*Induction and Modulation of IL-1, IL-6, and TNF by LPS and Its Analogs*	Dr. Hans-Dieter Flad
11:30 - 12:10	*LPS Processing by Phagocytes: An Update*	Dr. Robert S. Munford
12:10 p.m. - 1:15 p.m.	Lunch	
1:30 p.m. - 5:10 p.m.	**Session IV** **Non-Specific Increases in Resistance**	*Chairperson:* Dr. Richard A. Proctor

Monday, April 8 (continued)

1:30 - 2:10	*Role of Purinoreceptor in Endotoxin Activation of Macrophages*	Dr. Richard A. Proctor
2:10 - 2:50	*Identification and Characterization of LPS Binding Proteins on Mammalian Cells*	Dr. Mei-Guey Lei
2:50 - 3:30	*Protective Effects of MPL in Models of Sepsis and Endotoxemia*	Dr. J. Terry Ulrich
3:30 p.m. - 3:50 p.m.	Coffee Break	
3:50 - 4:30	*MPL as Prophylaxis for Surgical Wound Infection: A Phase I-II Study*	Dr. David Beatty
4:30 - 5:10	*Induction of Endotoxin Tolerance by Non-Toxic Derivatives of Lipid A*	Dr. Mark E. Astiz
7:30 p.m.	**Evening Lecture** *Molecular Mechanisms of Intracellular Signalling*	Dr. Masayasu Nakano

Tuesday, April 9

8:30 a.m. - 12:10 a.m.	**Session V** **Current Vaccine Strategies Using Lipid A Derivatives**	*Chairperson:* Dr. Barbara Detrick
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8:50 - 9:35	*Development of Synthetic Lipid A Analogue Vaccine*	Dr. Alan S. Cross
9:35 - 10:20	*Adjuvant Effects of Liposomal Lipid A: Human Vaccine Induces Antibodies to Both Malaria Antigen and Lipid A*	Dr. Carl R. Alving
10:20 a.m. - 10:40 a.m.	Coffee Break	
10:40 - 11:25	*An Effective Vaccine for Syphilis: The Importance of Adjuvant Action of MPL in Reversing Immunosuppression*	Dr. Thomas Fitzgerald
11:25 - 12:10	*Evaluation of MPL as Adjuvant for Human Immunization: Enhanced Antibody Responses with Concurrent Injection of MPL and Polysaccharide Conjugates*	Dr. Sarvamangala Devi

Keynote Address

Current Developments in AIDS Vaccine Adjuvant Research

Saturday, April 6 • 7:30 p.m. - 8:00 p.m.

Dr. Wayne C. Koff

National Institute of Allergy and Infectious Diseases, NIH • Bethesda, MD

Session I

New Advances in the Structural and Functional Relationships of Lipid A and Its Analogs (Part I)

Sunday, April 7 • 8:30 a.m. - 12:00 noon

Speakers' Summaries (in order of presentation)

LIPID A ANALOGS AIMING AT PREVENTION OF THE DELETERIOUS EFFECTS OF ENDOTOXIN: STRUCTURE-ACTIVITY RELATIONSHIPS.

P. Stuetz, SANDOZ Forschungsinstitut, Vienna, Austria.

Despite the advent of potent new antibiotics with a broad spectrum of activity, overall morbidity and lethality in patients with Gram-negative bacteremia have not changed over the years. At present, two different approaches are being pursued with lipid A analogs to prevent the deleterious effects of endotoxin whose dominant role in septic shock has been well established.

Highly lipophilic lipid A analogs, i.e. monosaccharidic or disaccharidic glucosamine-phosphates that contain at least 3 N,O-bound long-chain fatty acids per glucosamine-phosphate backbone have the ability to enhance non-specific resistance to bacterial and fungal infections, presumably by stimulating the secretion of colony stimulating factors, and by priming for oxidative burst and bacterial killing of macrophages and neutrophils. These events are paralleled by the development of so-called early-phase tolerance to endotoxin, a reversible state of hyporesponsiveness against otherwise lethal doses of LPS. Paradoxically, repeated administration of this class of compounds potentiates resistance to infections, but also tolerance to endotoxin even further and for a longer period of time. From the limited information available it appears that this class of compounds, like LPS or lipid A are also good adjuvants. Possible modes of action underlying these effects will be discussed.

The second class represent much more polar monosaccharidic or disaccharidic lipid A analogs with a maximum of 2.5 N,O.bound long-chain fatty acid residues per glucosamine-phosphate which can directly block LPS-effector mechanisms, presumably by competing with LPS-binding sites. These compounds also protect against a lethal LPS-dose in rodents. Their adjuvant effects have not been investigated so far, but in the prolymphoid 70/3Z cell line, prototype compounds are able to effectively block the LPS-induced differentiation into mature B cells.

Synthetic lipid A analogs with monosaccharide backbone, which are nonpyrogenic with various biological activities

Motohiro Matsuura[1], Makoto Kiso[2] and Akira Hasegawa[2]
[1]Jichi Medical School, Tochigi, Japan and [2]Gifu University, Gifu, Japan.

A typical lipid A (Escherichia coli type) is composed of glucosamine disaccharide backbone with 1,4'-bisphosphate and hexaacyl substituents at the 2, 2', 3 and 3' positions. We have synthesized lipid A analogs with monosaccharide backbone carrying phosphate and acyl substituents, and their biological activities examined. Analogs possessing monophosphate and triacyl substituents to monoglucosamine backbone were generally active. A representative analog (GLA-60), which exhibits various immunomodulating activities such as induction of mediators, activation of B cells or macrophages, enhancement of nonspecific resistance and so on, has a 3-hydroxytetradecanoyl (C_{14}-OH), a 3-tetradecanoyloxytetradecanoyl (C_{14}-O-(C_{14})) and a phosphate groups at the 2, 3 and 4 positions of a glucosamine backbone, respectively. An analog GLA-63 with a C_{14}-O-(C_{12}) group instead of a C_{14}-O-(C_{14}) group in GLA-60 exhibited as high activities as GLA-60, but the activities of GLA-64 with a C_{14}-O-(C_{16}) group diminished markedly. Moreover, analogs with a tetradecanoyl (C_{14}) and a C_{14}-O-(C_{16}) groups as acyl substituents at the 2 and 3 positions (GLA-69 and GLA-38) were nearly nonactive. Tolerance inducing activity against lethal toxicity of LPS was, however, observed even in such scarcely active analogs. The most interesting point in biological activities of the synthetic monosaccharide analogs is that neither pyrogenicity nor Shwartzman reactivity is exhibited even by the highly active analogs such as GLA-60 and GLA-63.

CURRENT STATUS OF THE STRUCTURE-FUNCTION RELATIONSHIP OF LIPID A

Kuni Takayama

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and
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Madison, Wisconsin 53706

The endotoxic moiety of the lipopolysaccharide (LPS) of Gram-negative bacteria is the lipid A. Since its "correct" structure was elucidated in 1983, many investigators have studied the structural basis for the numerous biological activities of lipid A. This included chemical synthesis of analogs. From all these studies, one can reach a simple conclusion that the most endotoxic lipid A structure is the hexaacyl diphosphoryl lipid A found in the LPS of the *Salmonella* strains and *Escherichia coli* [β(1→6)glucosamine disaccharide containing phosphate groups at the 1- and 4′-positions, HOC_{14} fatty acids at the 2- and 3-positions, $C_{12}OC_{14}$ at the 2′-position, and $C_{14}OC_{14}$ at the 3′-position]. We call this the model "toxic" lipid A. A departure from this basic structure leads to lower biological activities. Nature modulates the biological activities of this toxic lipid A by controlling (i) the number of phosphate groups in the disaccharide, (ii) the number, kind, and size of the hydroxy and normal fatty acids in the disaccharide, and (iii) the polar substituents to be added to the basic structure. Another variation that nature has devised is to use 2,3-diamino-2,3-dideoxyglucose in the disaccharide backbone.

A model "nontoxic" lipid A can be obtained from the LPS of *Rhodopseudomonas sphaeroides*. It is closely related structurally to the model toxic lipid A. We will show that by merely reducing the fatty acid content from six to five and lowering the size of the hydroxy fatty acids at the 3- and 3′-positions from C_{14} to C_{10}, the toxic lipid A is converted to nontoxic lipid A. These results emphasize the strong influence of specific hydroxy fatty acids in the lipid A moiety of LPS in determining its endotoxic property.

REGULATION OF NITRIC OXIDE SYNTHESIS BY LIPID A DERIVATIVES

G.L. Gustafson and M.J. Rhodes
Ribi ImmunoChem Research, Inc., Hamilton, MT 59840

Recent studies in several laboratories have shown that LPS can induce the expression of nitric oxide (NO) synthetase in macrophages as well as in tissue cells of animals. Expression of this enzyme in macrophages is a protective response since macrophage production of NO enhances antimicrobial and antitumor activities. However, expression in tissue cells is detrimental since tissue NO is a mediator of endotoxin shock. In the present work the potentials of LPS and lipid A derivatives to regulate NO synthesis were studied 1) in cultured murine peritoneal macrophages (macrophage model) and 2) in intact mice (systemic model). NO production was quantified indirectly by measuring the accumulations of nitrite and nitrate in cell culture media and in blood samples. Lipid A derivatives were compared with regard to their activities to stimulate NO production and to block LPS-stimulated NO production. The regulation of NO synthesis in the systemic model differed in many ways from the regulation of macrophage NO synthesis. These included 1) differences in structure/activity relationships for lipid A derivatives between the two models, 2) differences in the kinetics of NO production, and 3) differences in the patterns of blocking of NO production by lipid A derivatives. Of special interest, it was found that monophosphoryl lipid A (MPL) did not enhance systemic production of NO, but that it did induce NO synthesis in cultured macrophages. Based on all of the differences observed, it was hypothesized that tissue cells rather than macrophages might be the major producers of NO in mice challenged systemically with LPS. The demonstrations that pretreatment of mice with MPL attenuated the ability of LPS to induce systemic NO production but did not itself induce NO production supported the view that MPL might have applications as a prophylactic drug for endotoxic shock.

Session II

New Advances in the Structural and Functional Relationships of Lipid A and Its Analogs (Part II)

Sunday, April 7 • 1:20 p.m. - 4:00 p.m.

Speakers' Summaries (in order of presentation)

OVEREXPRESSION OF KDO TRANSFERASE ACTIVITY. C. R. H. Raetz. Merck Sharp and Dohme, Rahway, NJ.

An autoradiographic assay applicable to colonies immobilized on filter paper was developed for obtaining temperature sensitive mutants of Escherichia coli defective in the transfer of 3-deoxy-D-manno-octulosonic acid (KDO) from CMP-KDO to a tetraacyldisaccharide-1,4'-bisphosphate precursor of lipid A, designated lipid IV_A. Cell-free extracts from two mutants found in a population of 30,000 mutagen treated cells showed normal KDO transferase activity when assayed at 30°C, but almost no activity at 42°C. The mutation was mapped by mating one of the mutants with different *Hfr* strains and analyzing genetic linkage of KDO transferase activity to selectable markers. The lesion was located to a position between 80-84 min on the E. coli chromosome. A plasmid from the Clarke and Carbon collection, pLC17-24, known to contain genes from the rfa region (81 min), was shown to overexpress KDO transferase activity 4-5 times and to correct the mutation when the plasmid was conjugated into the mutant strains. The KDO transferase gene, designated kdtA, was subcloned from pLC17-24 into a multicopy vector. The resulting plasmid, pCL3, overproduced transferase activity ~100-fold. The kdtA gene was shown to code for a 43 kDa polypeptide, as judged by radiolabeling of minicells. Its DNA sequence was determined. The results demonstrate that overexpression of this single gene product greatly stimulates the incorporation of two stereochemically distinct KDO residues during LPS biosynthesis in extracts of E. coli.

Modulation of Antibody Isotype by Lipopolysaccharides in Combination With Nonionic Block Copolymers

Robert L. Hunter
Department of Pathology,
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Mycobacteriology Laboratory,
William S. Middleton Memorial Veterans Hospital,
Madison, WI 53705;

Nonionic block copolymers and lipopolysaccharides are both effective immunologic adjuvants which are thought to act via distinct mechanisms. We hypothesized that they might produce synergistic effects when used together. We prepared a series of LPS preparations ranging from the smallest precursor, lipid X through complete LPS with O-polysaccharide chains. Three preparations with reduced toxicity, monophosphoryl lipid A, partially hydrolyzed Ra-LPS and LPS of *R. sphaeroides* were also utilized. All LPS preparations except the smallest were effective adjuvants for inducing early antibody responses to TNP-HEA when injected in squalane-in-water emulsions with copolymer L141. Only the larger LPS preparations induced sustained antibody responses. By itself, emulsions of copolymer L141 induced a predominant IgG1 antibody isotype response with lesser amounts of IgG2a and IgG2b. Surprisingly, all of the LPS preparations tested increased the proportion of IgG2 isotypes even though some had little effect on overall titers. The detoxified Ra-LPS (Ra-detox) was the most effective preparation for both increasing antibody titers and inducing the desirable IgG2a and IgG2b isotypes. These results demonstrate that the combination of LPS and block polymer adjuvants can produce synergistic effects without unacceptable toxicities.

Lipid A-like Molecules Antagonize the Effects of Endotoxins on Human Monocytes and Neutrophils. Douglas Golenbock, M.D., Boston City Hospital, Boston MA. Lipopolysaccharide (LPS) is implicated as the bacterial product responsible for the clinical syndrome of gram-negative bacterial septicemia. Although the lipid A domain of LPS appears to be responsible for the toxicity of endotoxin, lipid A from the photosynthetic bacterium *Rhodobacter sphaeroides* (RSLA), two disaccharide precursors of lipid A from enteric bacteria termed lipid IV_A and KDO_2-lipid IV_A, and the monosaccharide lipid A precursor lipid X have little stimulatory activity on human cells. Using human mononuclear and polymorphonuclear phagocytes, we have shown that these molecules are specific antagonists of LPS. Complete, apparently competitive, inhibition of LPS activity of both monocytes and neutrophils was possible at a 10-fold excess of the disaccharide antagonists, as judged by measuring the release of cytokines and PGE_2 from monocytes or the surface expression of CD11b/CD18 (CR3). The LPS inhibitors had no effect on the stimulation of monocytes by purified protein derivative from *Mycobacterium tuberculosis* or heat-killed *Staphylococcus aureus* nor on neutrophil CR3 upregulation by TNFα, formyl-met-leu-phe or phorbol myristate acetate. Lipid X had no activity when tested against monocytes (up to a concentration of 1 μg/ml) and did not inhibit LPS-induction of TNFα or PGE_2 release. At high concentrations (10 μg/ml), lipid X acted as an antagonist when tested against LPS-induced PMN responses. The effect of the disaccharide inhibitors could not be removed when treated monocytes or neutrophils were extensively washed and then exposed to LPS. In contrast, the effects of lipid X could easily be washed away from treated PMN suggesting either that lipid X rapidly dissociates from neutrophil LPS receptors or inhibits LPS through non-competitive means (such as sequestration of LPS). The lipid A-based inhibitors may be useful in the design of novel therapeutic agents for gram-negative bacterial septicemia.

Host-Responses to LPS and Bacteria.

C.Galanos and M.A.Freudenberg
Max-Planck-Institut für Immunbiologie, 7800 Freiburg, FRG

The lethal toxicity of LPS is mediated by macrophages through tumor necrosis factor α (TNFα) which is probably the primary mediator of the lethal activity of LPS. LPS lethality may be completely inhibited by anti-TNFα antibodies. Smooth and rough (Ra-Re) LPS as well as free enterobacterial lipid A induce lethal effects in D-galactosamine treated mice. Synthetic hexaacylated lipid A (506) and monophosphorylated tetraacylated lipid A precursor Ia (406) were also active. The ability of synthetic compounds 506 and 406 to stimulate macrophages to produce TNFα in vitro was determined and compared to that of LPS. The experiments revealed that all 3 agents exhibited similar effects in cultures of mouse macrophages. However only LPS, but not compound 506 or 406, stimulated TNFα in cultures of human macrophages. It indicates that different structural prerequisites may be required in different animal species and/or different macrophage populations may respond differently to different LPS substractures.
Like lethality also the tolerance-inducing properties of LPS are mediated by macrophages through TNFα. Pretreatment of mice with minute amounts of LPS or TNFα will protect them to a subsequent challenge with lethal amounts of either agent in D-GalN sensitized mice.
TNFα is the mediator of the lethal activity of gram-negative and gram-positive bacteria. While several mg of purified LPS will not kill D-GalN treated LPS-resistant mice, a few µg of the same bacteria do so very effectively. This is evidence for the presence of shock inducing components, other than LPS, in gram-negative bacteria. Killed gram-positive bacteria also exhibit lethal effects in D-GalN sensitized LPS-sensitive and -resistant mice. The lethal activity of gram-negative and -positive bacteria may be inhibited by anti-TNFα antibodies. This is evidence that TNFα is a common mediator of the lethal activity of gram-negative and - positive bacteria. Minute amounts of both types of bacteria when used to pretreate mice, induce non-specific resistance to LPS and to their own lethal effects, and vice versa.

Evening Workshop

Implications and Consequences of Lipid A Administration

Sunday, April 7 • 7:30 p.m.

Session III

Cellular Mechanisms and Cytokines

Monday, April 8 • 8:30 a.m. - 12:00 noon

Speakers' Summaries (in order of presentation)

Inactivation of Suppressor T Cell (Ts) Activity by Monophosphoryl Lipid A (MPL) and its Structural Analogs (P.J. Baker)

Previous studies have shown that treatment with MPL abrogates the expression of Ts activity without adversely effecting other T cell functions (e.g., helper and amplifier T cell activity). LPS-responsive (LPS^r) and LPS-defective (LPS^d) strains of C3H mice do not differ in their capacity to make an antibody response to Type III pneumococcal polysaccharide (SSS-III) or in the degree of Ts activity generated after exposure to this antigen; however, treatment with MPL abolishes the expression of Ts function in LPS^r -- but not in LPS^d -- mice. This suggests that these mice may differ with respect to the presence of (a) a cell-surface receptor needed for the specific binding of MPL to activated Ts, and/or (b) an unique biochemical pathway in Ts that is sensitive to MPL. Several synthetic analogs of lipid A were tested to obtain relevant information on this issue. The results obtained suggests that the minimal structural unit required to produce the effects described is a diglucoseamine to which must be attached at least 5 fatty acids with perhaps one of them being an acyloxyacyl fatty acid at the 2' or 3' position. The ability of MPL or its analogs to inactivate Ts function appears to be independent of its ability to polyclonally activate B cells and the number of phosphate groups present.

IMMUNOREGULATION BY LIPID A ANALOGS. Arthur G. Johnson, Marilyn Odean, and Akira Hasegawa. University of Minnesota, Duluth, MN and Gifu University, Gifu, Japan.

LPS can either enhance or suppress the immune response depending on its time of administration relative to antigen. Our current research is directed towards (a) determining the minimal structural requirements responsible for enhanced antibody titers and/or their down regulation, and (b) identifying the target cell(s) and mediator(s) initiating both of these regulatory phenomena. In earlier studies with monophosphoryl lipid A (Ribi) we have implicated a Th_1 cell as the initial target cell and secretion of interferon gamma as vital to the enhancing action. In addition, suppression of antibody was also induced by MPL when given before antigen. Thus, neither KDO nor the reducing sugar phosphate is required for these actions.

Recently, a number of glycolipids representing the non-reducing moiety of lipid A have been synthesized and characterized by Hasegawa, et al. We have initiated testing of three of these analogs for their ability to suppress PFC formation when given before antigen. Our findings to date indicate that 25 µg of GLA 27 and GLA 60 injected ip effectively suppressed the murine PFC response. However compound 47, was ineffective, even at twice the dosage. Examination of their structural differences revealed that addition of a C_{14}-O-acyl group to the C_{14} fatty acid at the R'3 site to GLA 27 (which then becomes GLA 47) inactivated the capacity to suppress PFC formation. However, keeping the O-acyl linkage on R'3 but removing it from the C_{14} fatty acid on R'2 (now GLA 60) once again permitted suppression to be expressed. No significant pattern emerged from the suppression induced by three other analogs.

EARLY ENDOTOXIN TOLERANCE: A MODEL FOR DIFFERENTIAL CYTOKINE INDUCTION AND GENE EXPRESSION BY LPS AND MPL.

Stefanie N. Vogel and Beth E. Henricson, Uniformed Services University of the Health Sciences, Bethesda, MD 20814 USA.

Previous studies have shown that both smooth lipopolysaccharide (LPS) and the non-toxic, lipid A derivative, monophosphoryl lipid A (MPL), are capable of inducing a state of "early endotoxin tolerance" (EET) in mice; however, one needs significantly more MPL than LPS to achieve a level of desensitization comparable to that induced by LPS. Since cytokines have been implicated in the induction of EET, we examined levels of cytokines induced by LPS vs. MPL and found that although comparable levels of CSF were induced by tolerizing doses of each, the levels of other cytokines (e.g., TNF, IL-6, and IFN) are markedly reduced in animals injected with MPL. An in vitro model of LPS desenstization was established to dissect the problem further. Briefly, the macrophage cell line, RAW264.7, or thioglycollate-elicited peritoneal exudate macrophages were exposed first to medium, LPS, or MPL for 20 hr and then "challenged" with LPS for an additional 20 hr and the level of TNF produced in the supernatants measured. As was observed in vivo, significantly more MPL was required to induce the same level of desensitization induced by LPS. Finally, induction of transcription of "early" endotoxin-induced genes was compared in macrophages treated with LPS vs. MPL. For seven genes examined, the concentration of MPL required to induce transcription of LPS-induced "early" gene expression ranged from 20- to 2500-fold that of LPS, depending on the individual RNA species analyzed. This approach should provide significant insights into the mechanisms by which LPS and MPL transmit their respective signals and result in toxic vs. beneficial effects in the host. (Supported by USUHS R07338 and NMRDC 63706.0095.001).

Induction and modulation of IL-1, IL-6, and TNF production by LPS and its partial structures

H.-D.Flad, Department of Immunology and Cell Biology, Forschungsinstitut Borstel, D-2061 Borstel, FRG

Bacterial lipopolysaccharides (LPS) consist of a hydrophilic heteropolysaccharide component and a covalently linked hydrophobic lipid portion termed lipid A. In studying the structure-function relationship of LPS and lipid A partial structures it could be established that S- and R-form LPS are very potent inducers of IL-1 and TNF in human monocytes in vitro, while synthetic S.minnesota heptaacyl lipid A (compound 516) and the monophosphorylated partial structures (compounds 504 and 505) were less active. Evidence is presented that the synthetic tetraacyl partial structure of E.coli lipid A (precursor Ia, compound 406) and the monosaccharide structure (lipid X, compound 401) do not induce IL-1, IL-6, and TNF, but modulate LPS-induced cytokine production. These compounds are able to suppress and/or delay LPS-induced TNF and IL-1β in a concentration-dependent fashion. Compound 406 is not able to inhibit phorbol-12-myristate-13-acetate (PMA)-induced TNF and IL-1 release, which suggests that its modulating effect is LPS-specific. Evidence is presented which indicates that the modulating effect of compound 406 on LPS-induced cytokine production is due to a competition of binding.

LPS PROCESSING BY PHAGOCYTES -- AN UPDATE. R. S. Munford. University of Texas Southwestern Medical Center, Dallas, TX.

Phagocytes process LPS principally by hydrolyzing the fatty acyl chains and phosphates from lipid A, leaving the saccharide chain (O-antigen) largely intact. Of the catalytic enzymes known to take part in LPS degradation by animal phagocytes, acyloxyacyl hydrolase (AOAH) has been most intensively studied. This enzyme removes the secondary acyl chains from LPS, producing a lipid A moiety that resembles the LPS precursor IVa and synthetic analog 406. In keeping with the reported bioactivities of these lipid A partial structures, enzymatically deacylated S. typhimurium, E. coli, P. aeruginosa, and H. influenzae LPSs are non-toxic (e.g., unable to stimulate human vascular endothelial cells) yet retain other biological activities, including murine spleen cell mitogenicity. Interestingly, Neisseria LPS is unable to elicit murine spleen cell mitogenesis and will inhibit the stimulatory ability of other LPSs in this assay system. It therefore appears that the contribution of the lipid A secondary acyl chains to the bioactivity of a given LPS cannot be predicted from the bioactivities of the existing lipid A analogs; possible explanations for this observation will be discussed.

Session IV

Non-Specific Increases in Resistance

Monday, April 8 • 1:30 p.m. - 4:45 p.m.

Speakers' Summaries (in order of presentation)

"Role of the Purinoreceptor in Endotoxin Activation of RAW264.7 Macrophages".

T. TANKE, H. RHIM, J-W. VAN DE LOO, P.S. LEVENTHAL, R.A. PROCTOR,* and P.J. BERTICS. Univ. of Wisc. Medical School, Madison, WI.

Studies of LPS-induced release of toxic mediators from macrophages have implicated G-proteins on the basis of pertussis toxin (PT) inhibition of IL-1 release. However, direct studies of G-protein activity have not been reported. Hence, we isolated membranes from RAW264.7 macrophages and studied GTPase activity as a measure of G-protein activation. Kinetic analyses showed maximal LPS-induced increase (3-fold) of velocity within 15 min that is enhanced by ATP, ADP, AMPPNP, ammonium sulfate, but is PT insensitive, even though PT was able to ribosylate a 40kDa protein. However, ADP doses that inhibit ATPase activity stimulated LPS-induced GTPase activity. Lipid X, which reduces LPS-lethality, dose-dependently blocked LPS-activation of the GTPase in the presence of ATP. These effects appear specific as other lipids, detergents, and glycosides neither stimulated nor inhibited LPS-induced GTPase activity. Thus, LPS may activate macrophages via coupling a G-protein to a membrane receptor, and this interaction may be modulated by ATP. As ATP infusion causes shock and death, LPS might cause toxicity by sensitizing cells to ambient ATP levels.

IDENTIFICATION AND CHARACTERIZATION OF LPS BINDING PROTEINS ON MAMMALIAN CELLS. M.-G. Lei, T.-Y. Chen, J. Halling, S. Field, L. Flebbe and D. C. Morrison. Univ. of Kansas Medical Center, Kansas City, KS 66103.

Studies from our laboratory have focused upon the identification and characterization of endotoxic lipopolysaccharide receptors expressed on mammalian lymphoreticular cells. These experiments have employed a photoactivatable disulfide reducible radioiodinated derivative of LPS synthesized by our laboratory (Wollenweber and Morrison, J. Biol. Chem., 260, 15068, 1985). Using S-LPS from E. coli 0111:B4 we identified a membrane glycoprotein of approximately 80 kDa on mouse lymphocytes and macrophages which manifest a number of properties consistent with it's functioning as a specific LPS receptor. Our studies suggested that binding specificity for this 80 kDa protein was for the lipid A region. We have recently confirmed this hypothesis by the synthesis of photoderivatized LPS from the deep rough S. minnesota Re chemotype LPS, where binding to an 80 kDa protein can readily be demonstrated. Extension of these studies to selected subpopulations of human peripheral blood cells has confirmed the existence of similar 80 kDa proteins on polymorphonuclear leukocytes, monocytes, B and T lymphocytes and platelets. These proteins are not detectable on human erythrocytes. Competitive inhibition studies readily demonstrate inhibition of binding to the 80 kDa protein with a variety of LPS and lipid A, including that from R.1 spheroides but not with a variety of peptidoglycans. We have recently begun characterization of a second LPS binding protein of approximate molecular mass 38 kDa. Of interest, photoderivatized binding of LPS to this protein is inhibited by both S and R chemotype LPS but not by purified Lipid A. The functional role of this 38 kDa protein remains to be defined (Supported by AI 23447).

Protective Effects of Monophosphoryl Lipid A in Models of Sepsis and Endotoxemia

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Monophosphoryl lipid A (MPL) and 3-0 deacylated MPL (3D-MPL) are prepared by partial degradation of Re LPS from Salmonella minnesota R595. These materials are greatly attenuated with respect to their toxic activities. A major focus in our laboratory has been to evaluate the ability of these substances to enhance non-specific resistance to bacterial challenge and to protect against lethal endotoxemia. Pretreatment of mice (I.P.) with 25-50 μg MPL or 3D-MPL results in maximal protection (80-100% survival) to Escherichia coli (5 x 10^6 CFU) given 48 hr. later. Increased non-specific resistance in this model was temporary. Less than 10% survival was observed if the challenge was given 5 days after MPL or 3D-MPL treatment. Experiments in which mice received multiple treatments with 3D-MPL prior to challenge indicated that a minimum of 6 days was required before re-treatment could elicit maximum protection. D-galactosamine (D-gal) treatment profoundly sensitizes mice to endotoxin/LPS. Using the D-gal model we have found that 3D-MPL pretreatment (I.V.) produces a hyporesponsive state to LPS that occurs within 6 hrs. and which persists for 48 hrs., with protection rapidly declining by 72 hrs. Multiple dosing studies with 3D-MPL given daily or every other day demonstrated that this hyporesponsive state to LPS challenge could be extended for up to 7 days. Tumor necrosis factor (TNFα) production by macrophages in response to LPS stimulation has been shown to mediate lethality in D-gal compromised mice. Changes in TNFα concentrations in mouse sera were measured following D-gal/LPS challenge. Mice pretreated with 3D-MPL produced only 30% as much serum TNFα as control mice when challenged with LPS. D-gal treatment of mice has also been shown to increase their sensitivity to the direct toxic/lethal effects of human recombinant TNFα (hrTNFα). We found that 3D-MPL pretreatment abrogates the toxicity of injected hrTNFα. We have conducted a series of studies in rabbits designed to evaluate the effect of 3D-MPL pretreatment on a subsequent lethal challenge (35 μg/kg) or pyrogenic challenge (40 ng/kg) of Salmonella abortus equi LPS. Pretreatment of rabbits I.V. with 3D-MPL over a non-pyrogenic dose range of 2.5 μg/kg to 12.5 μg/kg provided significant protection (80-100%) to a lethal challenge of LPS given 24 hrs. later. However, pretreatment with non-pyrogenic doses of 3D-MPL (≤12.5 μg/kg) did not significantly alter the biphasic fever response to a pyrogenic (40 ng/kg) challenge of LPS. Of interest was that rabbits treated with doses of 3D-MPL in excess of 50 μg/kg exhibited a monophasic fever (peak at 1.0 hr.), yet when challenged 24 hrs. later with 40 ng/kg LPS they appeared to be refractile with respect to the second fever peak typically seen at 2.5-3.0 hr. in control rabbits. These studies taken collectively have suggested to us that 3D-MPL may have prophylactic value in ameliorating the problem of sepsis and septic shock in clinical medicine.

Monophosphoryl Lipid A as Prophylaxis for Surgical Wound Infection
A Phase I/II Study

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This study was undertaken to examine the effect of monophosphoryl lipid A (MPL), an attenuated analogue of toxic lipid A, as a nonspecific pre-operative immune stimulant in patients undergoing complex surgical resections for head and neck cancer. MPL was given 24-48h prior to the surgical procedure. All patients received prophylactic cephoxitin and gentamicin for 3 days beginning 8 hours prior to surgery. Patients were observed for toxicity, wound complications and systemic complications. Hematological, biochemical and immunological blood tests were performed pre-MPL infusion: 2h and 24h post-MPL: pre-surgery: and 3, 7 and 28 days after surgery.

Escalating doses of MPL were given to 19 patients: 10 $\mu g/m^2$ (2 pt), 35 $\mu g/m^2$ (2 pt) and 100 $\mu g/m^2$ (15 pt). At 100 $\mu g/m^2$, 60% of patients (9/15) had MPL related toxicity including chills and rigor (8/15), fever (7/15), headache (4/15), muscle pain (2/15), lethargy (1/15) and weakness (1/15). There were 14 grade 1, 9 grade 2, and 1 grade 3 toxicities. To decrease toxicity during the study, the dose of MPL was limited to the greater of 200 μg or 2.5 $\mu g/kg$ ideal body weight in patients with body surface area over $2m^2$.

At 100 $\mu g/m^2$ MPL, 7/15 patients had surgical wound complications including decreased flap viability (6/15), erythema (6/15), induration (4/15), infection (2/15), fistula (1/15), skin contamination (1/15) and dehiscence (1/15). Postoperatively, all patients (15/15) had fever and one patient had respiratory tract infection. There were no cardiac or renal complications. No association was noted between MPL toxicity, and local or systemic complications.

Standard hematological and biochemical tests were unaffected by MPL except for neutrophil count. Juvenile neutrophils (bands) were increased from <1% to 6% at 2h post-MPL and mature neutrophils from 66% to 78%. By 24h they had returned to normal. The surgical procedure resulted in a rise in neutrophils, platelets, fibrinogen and liver function tests.

Levels of immunoglobulin (IgG, IgA, IgM), β_2 microglobulin, complement (C_{3c}, C_4), lymphocyte markers (CD2, CD4, CD8, CD20, CD25, DR, NK), monocyte/macrophage adherence and phagocytosis, monocyte superoxide dysmutase, tetanus toxoid recall and the cytokines IL-2, GM-CSF and γ-INF were not affected by MPL infusion. The levels of serum CRP were elevated by both MPL and surgery. Serum IL-6 and TNF-α were normal (<10pg/ml) pre-MPL and 24h post-MPL. At 2h post-MPL IL-6 was elevated in 11/15 patients (358 $\pm$ 109 $\mu g/ml$) and TNF-α was elevated in 8/15 patients (32.3 $\pm$ 9.3 $\mu g/ml$). Levels returned to normal by 24h post-MPL. Correlation was noted between IL-6 and TNF-α levels, and between these cytokines and toxicity. Neopterin levels were increased at 24h post-MPL in 13/15 patients.

In conclusion, prophylactic MPL was safely administered prior to major surgical procedures and resulted in non-specific indications of an immune response and increased levels of cytokines associated with macrophage activation. Confirmation that prophylactic MPL significantly decreases the incidence of local wound sepsis would require a large multi-center trial.

Induction of Endotoxin Tolerance by Nontoxic Derivatives of Lipid A

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Several nontoxic derivatives of endotoxin (LPS) and lipid A have been recently isolated. These substances are significantly less toxic than the parent molecule yet maintain the beneficial immunomodulatory activities of endotoxin. We have been particularly interested in the ability of these substances to modify the systemic toxicity of endotoxin and to induce endotoxin tolerance.

Monophosphoryl lipid A (MPL) is a nontoxic derivative of lipid A which lacks a phosphate group at the reducing end of the molecule. The ability of MPL to modulate endotoxin activity was examined in several studies.

In initial studies, rats were pretreated with *S. minnesota* monophosphoryl lipid A fifteen minutes prior to endotoxin infusion. A significant attenuation of the decrease in arterial pressure and decrease in cardiac output was observed in MPL treated animals. Mean arterial pressure (MAP) decreased from 134 mmHg to 90 mmHg and cardiac output (CO) decreased from 356 ml/kg/min to 229 ml/kg/min in LPS animals while the animals pretreated with MPL showed no significant changes in MAP or CO.

In a subsequent study effect of MPL on LPS induced priming of human neutrophil superoxide radical production was examined. In contrast to LPS, MPL exhibited no neutrophil priming activity in concentrations ranging from 1 ng/ml to 10^3 ng/ml. Incubation of neutrophils with MPL prior to LPS resulted in dose-dependent inhibition of LPS-inducing neutrophil priming.

The induction of MPL was also examined in large and small animals models. In rats, exposure to MPL 48 hours prior to lethal endotoxin infusion produced 100%.

Seventy-two hour survival as compared to 100% mortality in animals pretreated with diluent control. Significant attenuation of decrease in arterial pressure and cardiac output were also observed. A similar study in a porcine model demonstrated that a pretreatment with MPL 48 hours prior to lethal endotoxemia produced 20% mortality as compared to 100% mortality in diluent controls. Hemodynamic changes following endotoxin infusion were significantly attenuated and tumor necrosis factor release was significantly reduced in the MPL treated animals.

The toxicity and ability of *R. sphaeroides* lipid A (RS) to induce tolerance was also studied. LPS and RS were infused in equal doses in rats. LPS resulted in significant decreases in cardiac output and arterial pressure whereas infusion of RS and was not associated with significant hemodynamic changes. Exposure of rats to RS forty-eight hours prior to lethal endotoxin infusion significantly decreased mortality from 100% in control animals to 20% of the tested animals.

These studies suggest that nontoxic derivatives of lipid A may be able to modify the systemic response to endotoxin by inducing endotoxin tolerance.

Evening Lecture

Molecular Mechanisms of Intracellular Signalling

Monday, April 8 • 7:15 p.m.

Dr. Masayasu Nakano

Jichi Medical School • Tochigi-Ken, Japan

MOLECULAR MECHANISMS OF INTRACELLULAR SIGNALLING IN MURINE MACROPHAGES BY LPS OR LIPID A ANALOGS

Masayasu Nakano, Department of Microbiology, Jichi Medical School, Tochigi-ken 329-04, Japan

Intracellular events in the peritoneal exudate macrophages of LPS-responsive C3H/He and LPS-low responsive C3H/HeJ mice after the stimulation with LPS or lipid A analogs were studied. LPS-induced IL-1 production by C3H/He macrophages seemed to be dependent on calmodulin-dependent pathway rather than the pathways dependent on protein-kinase C, A, or G. LPS induces phosphorylation of a distinct set of substrate proteins in the C3H/He, but not C3H/HeJ, macrophages prior to final LPS-induced cellular functions such as IL-1 or TNF production. The phosphorylated proteins (pp) were located in either a cytosol fraction (pp65 & 28) or a membrane fraction (pp58, 43,33, & 19) or in both fractions (pp105 & 75). Among the pp, pp65 was the most heavily phosphorylated cytosolic soluble protein, and was a substrate for serine kinase. When the macrophages were stimulated with biologically active lipid A analogs, the patterns of phosphorylation were similar to that of the macrophages stimulated with LPS. However, when the macrophages were stimulated with biologically non-active lipid A analogs, some of the pp were not observed.

Session V

Current Vaccine Strategies Using Lipid A Derivatives

Tuesday, April 9 • 8:30 a.m. - 11:25 a.m.

Speakers' Summaries (in order of presentation)

Abstract

Development of a Synthetic Lipid A Analogue Vaccine. Bhattacharjee AK, Drabick J, Wright CD, Sadoff JC, Cross AS. Department of Bacterial Diseases, WRAIR, Washington DC and Univax Biologics, Inc, Rockville, MD

The mortality from sepsis due to Gram-negative bacteria remains high despite the availability of potent antimicrobial agents. The recently reported success of a human MAb to lipid A, HA-1A, derived from a patient immunized with a J5 vaccine, suggests that immunotherapy directed at lipid A may be an important supplement to the conventional care of septic patients. Bieber et al. demonstrated that the binding of anti-J5 MAb to lipid A is inhibited by gentiobiose octaacetate. Consequently we constructed a synthetic lipid A analogue conjugate vaccine by coupling gentiobiose heptaacetate (GHA) to a protein carrier, succinylated diphtheria toxoid, through spacer compounds. This vaccine bound to the anti-lipid A MAb in ELISA assay, and induced antibody to GHA in rabbits and mice (>2500 ELISA antibody units [U]), but less antibody to J5 LPS (127 U v. 15 U in unimmunized rabbits) or to lipid A (100 U v. 25 U in unimmunized rabbits). Human antibody to lipid A derived from a volunteer immunized with monophosphoryl lipid A in a liposome, also bound to GHA (>675 units). A functionally-active lapine anti-J5 Ab bound to J5 LPS and lipid A (>2900 units) but not to GHA (16 units). Functional activity of anti-GHA Ab was demonstrated in a Limulus lysate neutralization test, and in protection against active and passive dermal Shwartzman reactions. Phase I testing with this vaccine will be initiated this year. These data also suggest there might be multiple epitopes to which functionally active Ab to lipid A may be directed.

ADJUVANT EFFECTS OF LIPOSOMAL LIPID A: HUMAN VACCINE INDUCES ANTIBODIES TO BOTH MALARIA ANTIGEN AND LIPID A

CARL R. ALVING, ROBERTA L. RICHARDS, JAMES E. EGAN, CLYDE SCHULTZ, DANIEL M. GORDON, AND LOUIS F. FRIES
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Liposomes containing lipid A served as carriers of a synthetic antigen for immunizing mice, rabbits, monkeys, and humans against sporozoites of *Plasmodium falciparum*. Liposomal native lipid A and liposomal monophosphoryl lipid A (MPL) had potent dose-dependent adjuvant properties. Although large amounts of lipid A were required for optimal activity, even very high doses of liposomal lipid A were nonpyrogenic in rabbits. An experimental vaccine was developed consisting of liposomes containing both MPL and a recombinant antigen ($R32NS1_{81}$) having epitopes from the repeat region of the circumsporozoite protein of *P. falciparum*. The liposomes were adsorbed with alum. A phase I safety trial of the vaccine in humans revealed no significant acute toxic effects and the vaccine was nonpyrogenic in humans even at an MPL dose of approximately 2.2 mg. High titers of IgG antibodies both to repeat region epitopes of the malaria antigen and to the MPL itself developed within two weeks after a single injection of the vaccine in humans. The highest dose of liposomal MPL induced the highest titers of antibodies to lipid A. Liposomes effectively blocked potential toxic effects but retained the adjuvant effects of lipid A. The vaccine consisting of alum-adsorbed liposomes containing high doses of MPL and encapsulated antigen appears to be safe and efficacious for inducing humoral immune responses in humans.

AN EFFECTIVE VACCINE FOR SYPHILIS: THE IMPORTANCE OF THE ADJUVANT ACTION OF MPL IN REVERSING IMMUNOSUPPRESSION. Dr. Tom Fitzgerald, University of Minnesota Duluth, MN. 55812

Abstract

Our research involves mechanisms of immunoregulation in chronic diseases, using syphilitic infection as the model. Following rabbit inoculation with Treponema pallidum, a number of immune functions are stimulated within the initial 10 days of infection. At this time, down-regulation of these functions becomes detectable, and levels of IL-1, IL-2, γ-interferon, and class II antigens are diminished. Macrophages and T-lymphocytes participate in this suppression through their secretion of PGE_2 and transforming growth factor (TGF). We have proposed that this down-regulation is premature and that residual treponemes then establish chronic infection. We have been able to reverse this down-regulation by using indomethacin to block macrophage suppression and monophosphoryl lipid A (MPL) to block T-suppression. Soluble factors including TGF are produced by activated lymphocytes. These factors inhibit Con A induced IL-2 synthesis and T-cell proliferation. MPL reverses both of these 2 suppressive effects. In addition, treponemes directly inhibit IL-2 generation and MPL reverses this defect. We have extended these findings to vaccine development. Our premise was that past vaccine failures were attributed to the ability of treponemes to induce down-regulation, thereby preventing immune stimulation. We have now succeeded in developing an effective vaccine by counter-acting these suppressive tendencies. Our protocol involves: cyclophosphamide day -2 (neutralizes natural suppressors and T-suppressors), inactivated T. pallidum plus Ribi adjuvant (MPL and trehelose dimycolate) day 0, MPL boost day +2 (neutralizes elicited T-suppressors), challenge with viable organisms day 29, and indomethacin day 29 to 36 (neutralizes macrophage suppressors). This unique vaccine approach of preventing suppression may have applications to other chronic diseases that involve macrophage suppression and/or T-cell suppression.

Dr. Tom Fitzgerald

University of Minnesota-Duluth

EVALUATION OF MONOPHOSPHORYL LIPID A (MPL) AS AN ADJUVANT FOR HUMAN IMMUNIZATION: ENHANCEMENT OF THE SERUM ANTIBODY RESPONSE IN YOUNG MICE TO POLYSACCHARIDE-PROTEIN CONJUGATES BY CONCURRENT INJECTION WITH MPL

Rachel Schneerson,[1] Ali Fattom,[1] Shousun C. Szu,[1] Dolores Bryla,[2] Ulrich,[3] Jon A. Rudbach,[3] Gerald Schiffman,[4] and John B. Robbins[1]
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Concurrent injection of monophosphoryl lipid A (MPL) in saline or as an oil-in-water emulsion (o/w) enhanced both the primary and secondary serum antibody responses to the capsular polysaccharide (CP) components of seven conjugates: the enhanced responses were Ag-specific. In contrast, MPL did not enhance the serum antibody response to 5 of the 6 unconjugated CPs. MPL and trehalose dimycolate (TDM) injected concurrently with the unconjugated Vi CP of *Salmonella typhi* (Vi) enhanced the serum antibody response to that Ag. MPL further enhanced the Vi antibody levels when injected with conjugates of this CP. The serum antibody responses to *Pseudomonas aeruginosa* exotoxin A (ETA), used as the carrier protein for the *Staphylococcus aureus* types 5 and 8 conjugates, were also enhanced by MPL. MPL in o/w was generally more effective than when administered in saline.

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Newer Aspects of the Adjuvant Action of Lipid-A and its Analogs

April 6-9, 1991

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